

Incidence of *Prevotella intermedia* and *Prevotella nigrescens* Carriage among Family Members with Subclinical Periodontal Disease

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We established a typing system for *Prevotella intermedia* and *Prevotella nigrescens* using the combination of PCR ribotyping and arbitrarily primed PCR (AP-PCR) fingerprinting and applied this system to the study of intrafamilial incidence of these species in the oral cavity. PCR ribotyping followed by subtyping by AP-PCR fingerprinting was applied to each type strain of *P. intermedia* and *P. nigrescens* and 54 isolates (32 isolates of *P. intermedia* and 24 isolates of *P. nigrescens*) from extraoral infections, resulting in an excellent discriminatory power (discrimination index, 0.99) for both species. A total of 18 subjects from six families, with the subjects from each family comprising the mother, the father, and a child who had subclinical early-stage to moderate adult periodontitis or simple gingivitis and who carried *P. intermedia* or *P. nigrescens*, or both, were enrolled in the study of intrafamilial carriage. When 20 colonies per specimen of subgingival plaque, if available, were picked from primary culture, 115 *P. intermedia* and 178 *P. nigrescens* isolates were recovered from the 18 subjects. Among the subjects studied, family members shared the same subtype strain(s) but non-family members did not. Multiple subtypes were found in 8 (57%) of the 14 *P. nigrescens*-positive subjects but in only 3 (27%) of the 11 *P. intermedia*-positive subjects; the difference was, however, not statistically significant ($P = 0.14$). These results suggest that the combination of PCR ribotyping and AP-PCR fingerprinting is well suited for the epidemiological study of *P. intermedia* and *P. nigrescens* and that each family seems to carry a distinct subtype(s) of these species.

Prevotella intermedia sensu lato is an obligatory anaerobic, black-pigmented, gram-negative rod that is frequently associated with periodontal disease: adult periodontitis, acute necrotizing ulcerative gingivitis, and pregnancy gingivitis. This organism is also involved in extraoral infections such as nasopharyngeal infection and intra-abdominal infection (10).

It was recognized that there is heterogeneity within *P. intermedia* strains in terms of serology and DNA homology. In 1992, a comprehensive DNA-DNA hybridization study proposed that *P. intermedia* be classified into two genospecies, *P. intermedia* and *Prevotella nigrescens* (18). Previous studies suggest that *P. intermedia* is likely to be more associated with periodontal sites, whereas *P. nigrescens* seems to be more frequently recovered from healthy gingivae, although the site specificities of these two species remain controversial (7, 11, 20).

It is of interest to investigate how humans get *P. intermedia* and *P. nigrescens* in the oral cavity and what the consequence of acquisition of these organisms is. So far there are few reports in the literature on the incidence or transmission of *P. intermedia* and *P. nigrescens* within families (11, 20). Establishment of efficient typing systems for *P. intermedia* and *P. nigrescens* is fundamental for epidemiological study of infecting or colonizing clones and for tracing the transmission of microorganisms from person to person. The techniques which have been used to type *P. intermedia* and *P. nigrescens* include sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2, 19), restriction endonuclease analysis (2, 15), ribotyping (15),

and arbitrarily primed PCR (AP-PCR) (10, 12). These techniques are time-consuming or have low levels of reproducibility. PCR ribotyping, which is expected to amplify the 16S and 23S spacer regions of bacterial rRNA genes by PCR, has been demonstrated to be a simple, rapid, and reliable typing technique (1, 8, 13). PCR ribotyping generates simple banding patterns and, thus, allows comparison of results obtained in separate PCR amplifications, whereas AP-PCR does not work in this manner. Recent studies have suggested that the use of multiple typing systems would be more reliable and would give a higher discriminatory power than any one typing system for the typing of microorganisms (3, 6, 17).

In this study, we established a typing system for *P. intermedia* and *P. nigrescens* using the combination of PCR ribotyping and AP-PCR fingerprinting and applied the established typing system to the study of intrafamilial carriage of these species in the oral cavity.

MATERIALS AND METHODS

Stock strains. The type strains *P. intermedia* ATCC 25611 and *P. nigrescens* JCM 6322 and 54 clinical strains of *P. intermedia* sensu lato isolated from patients with oral, otopharyngeal, and intra-abdominal infections at the Institute of Anaerobic Bacteriology, Gifu University School of Medicine, Gifu, Japan, were used to determine the discriminatory power of a typing system that combined PCR ribotyping with AP-PCR fingerprinting. *P. intermedia* sensu lato was identified on the basis of Gram staining, black-pigmented colony formation on anaerobic blood agar, and the results obtained with the Rapid ID 32A system (bioMérieux, Marcy-l'Etoile, France). Identification of *P. intermedia* and *P. nigrescens* was done by the PCR technique described below.

Subjects. We first studied an individual (a mother, a father, or a child) who visited a dental clinic due to dental diseases other than periodontal diseases and who was found to have early-stage to moderate adult periodontitis or simple gingivitis with a pathologically deepened periodontal pocket of ≤ 6 mm. Then, we asked the remaining family members to be enrolled in this study in order to cover all family members (the mother, the father, and the child). Eventually, this study enrolled 18 families; the periodontal diseases were subclinical for all subjects

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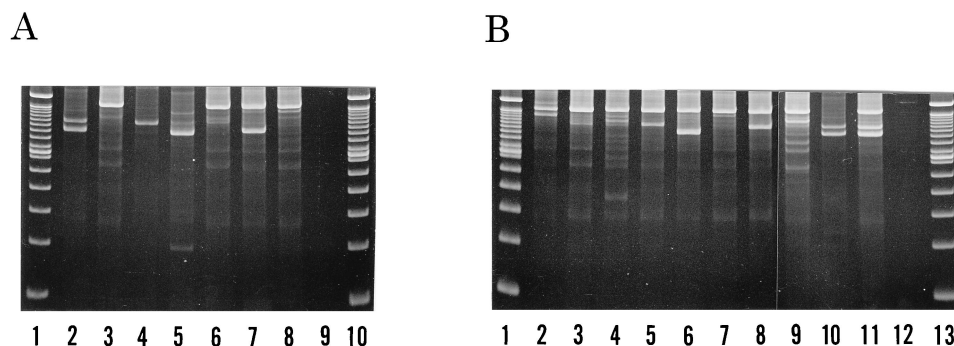


FIG. 1. PCR ribotyping patterns of *P. intermedia* (A) and *P. nigrescens* (B). (A) Lanes 1 and 10, 100-bp DNA ladder; lanes 2 to 8, *P. intermedia* types A to G, respectively; lane 9, negative control without DNA. (B) Lanes 1 and 13, 100-bp DNA ladder; lanes 2 to 11, *P. nigrescens* types A and E to M, respectively; lane 12, negative control without DNA.

studied, and no particular treatment for the periodontal disease other than tooth brushing was required. None of the subjects enrolled in the study had systemic diseases.

Bacterial sampling, culture, and isolation. The supragingival dental plaque was removed with sterile cotton, and the tooth surface was dried with compressed air to prevent contamination with saliva. With exclusion of moisture in the mouth with sterile cotton rolls, subgingival plaque was collected from the most inflamed sites by inserting a sterile no. 60 paperpoint into the periodontal pocket for 30 s (14). A plaque sample was inoculated onto each of Brucella HK blood agar (Kyokuto Seiyaku, Tokyo, Japan) and paromomycin-vancomycin Brucella HK blood agar (Kyokuto). The inoculated media were immediately incubated in an anaerobic environment generated with the AnaeroPack system (Mitsubishi Gas Chemical, Tokyo, Japan) for 3 or 4 days. Twenty black-pigmented colonies per specimen, if available, were subcultured onto Brucella HK blood agar and were subjected to the PCR technique (4) described below for the identification of the isolates as *P. intermedia* or *P. nigrescens*.

DNA extraction. Three- to 4-day-old cultures on Brucella HK blood agar were suspended in 500 μ l of lysis buffer (50 mM Tris-hydrochloride [pH 8.0], 5 mM EDTA, 50 mM sodium chloride, 1 mg of proteinase K per ml, 10% sodium dodecyl sulfate), and the mixture was incubated for 45 min at 56°C. After centrifugation at 14,000 rpm (15,000 \times g) for 5 min, DNA was extracted twice with 70% phenol-water-chloroform (Perkin-Elmer Applied Biosystems, Foster City, Calif.), adjusted to pH 7.6 with 1 M Tris-hydrochloride (pH 8.3), and precipitated with an equal volume of isopropyl alcohol at -20°C . Following centrifugation at 14,000 rpm (15,000 \times g) for 10 min, a DNA pellet was rinsed with ice-cold 70% ethanol and was resuspended in distilled water. The amount of extracted DNA was determined with a spectrophotometer (Gene Quant II; Pharmacia Biotech, Uppsala, Sweden), and the DNA sample was prepared to a concentration of approximately 1,300 ng/ml for PCR amplification.

PCR amplification for identification of *P. intermedia* and *P. nigrescens*. The PCR primers used for the identification of *P. intermedia* and *P. nigrescens* have been described by Guillot and Mouton (4); Pi754-1 (5'-CAGCACCACAACG ATATGA-3') and Pi754-2 (5'-TTCCATCTTCTCTGCTGTC-3') were used for *P. intermedia* and Pn1100-1 (5'-TTATGTTACCGTTATGATGGAAG-3') and Pn1100-2 (5'-ATGGCGAAATAGGAATGAAAGTTA-3') were used for *P. nigrescens*. PCR amplification was performed as described previously (16). In brief, DNA amplification was performed in a 30- μ l reaction mixture consisting of 10 mM Tris-hydrochloride (pH 9.0), 50 mM potassium chloride, 2.5 mM magnesium chloride, each deoxynucleoside triphosphate at a concentration of 200 μ M, and 0.75 U of *Taq* DNA polymerase (Pharmacia Biotech). DNA amplification was done for 35 cycles in a DNA thermal cycler 480 (Perkin-Elmer Applied Biosystems). The PCR cycle included denaturation for 20 s at 95°C and primer annealing-extension for 2 min at 50°C. As a final step, a 5-min extension was done at 74°C. The reaction products were subjected to polyacrylamide gel electrophoresis with a 5% polyacrylamide gel and a 100-bp DNA ladder (GIBCO BRL, Life Technologies, Rockville, Md.) and were visualized under UV transillumination following ethidium bromide staining.

PCR ribotyping and AP-PCR for *P. intermedia* and *P. nigrescens*. The primers used for PCR ribotyping were Primer-1 (5'-TTGTACACACCGCCGTC-3') and Primer-pin2 (5'-GGTACCTTAGATGTTTCAC-3'); Primer-1 is a primer used for PCR ribotyping of *Burkholderia cepacia* (9). PCR was run for 35 cycles as follows: denaturation for 1 min at 95°C, annealing for 1 min at 53°C, and extension for 2 min at 74°C. A final extension was done for 5 min at 74°C.

AP-PCR was carried out with the primer set of ERIC1R (5'-ATGTAAGCT CCTGGGGATTAC-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGC G-3') (21). DNA amplification was performed for 35 cycles, with each cycle comprising 1 min at 95°C, 1 min at 45°C, and 2 min at 74°C, with a single final extension step for 5 min at 74°C.

The PCR amplicons were resolved by a polyacrylamide gel electrophoresis

with a 4% polyacrylamide gel. The banding patterns were analyzed with Advanced Quantifier 1-D Match version 2.2.0 for Windows software (Genomic Solutions Inc., Ann Arbor, Mich.) to obtain the percent similarity of electrophoresis mobilities with the dendrogram of averages (unweighted pair group method with arithmetic means) algorithm.

Statistical evaluation for discriminatory power. The index of discriminatory power (D) was obtained by the following equation described by Hunter (5):

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^s X_j(X_j - 1)$$

where s is the number of the types, x_j is the number of population members falling into the j th type, and N is the size of the population.

Data analysis. For statistical analysis Fisher's exact probability test was performed with StatView 4.0 (Abacus Concepts, Berkeley, Calif.).

RESULTS

PCR ribotyping of stock strains of *P. intermedia* and *P. nigrescens*. Of 54 stock strains of *P. intermedia* sensu lato, 31 (57.4%) were identified as *P. intermedia* and 23 (42.6%) were identified as *P. nigrescens* by PCR amplification (data not shown).

PCR ribotyping was applied to *P. intermedia* ATCC 25611, *P. nigrescens* JCM 6322, and 54 stock strains (31 *P. intermedia* strains and 23 *P. nigrescens* strains). Since amplicons smaller than 600 bp were poorly reproducible, we analyzed a few reproducible major bands larger than 600 bp. Two or three DNA products were generated by most strains (Fig. 1).

The dendrograms for PCR ribotyping of *P. intermedia* and *P. nigrescens* are constructed separately (Fig. 2). Although the type strain and 31 stock strains of *P. intermedia* were grouped into seven types (Fig. 2A), the discrimination index was as low as 0.44 since type A is the most predominant type, with 24 strains (75%) being this type. Twenty-four *P. nigrescens* strains including the type strain were differentiated into 10 types (Fig. 2B), resulting in a discrimination index of 0.82. Type E was the most prevalent; 42% of the 24 strains tested were this type. Types A, E, F, and G were found in two species (Fig. 2).

Subtyping of stock strains by AP-PCR. To generate a higher discriminatory capability, the strains were further subtyped by AP-PCR fingerprinting. All strains of the same PCR ribotyping type were analyzed by one run of AP-PCR to evade the run-to-run variations in PCR amplification efficiencies. Strains were subtyped by duplicate AP-PCR. A representative result of the AP-PCR patterns of *P. intermedia* type A strains is shown in Fig. 3. Of the *P. intermedia* strains, 24 of type A and 3 of type B were separated into 20 and 3 subtypes, respectively (data not shown). Of the *P. nigrescens* strains, 10 type E strains

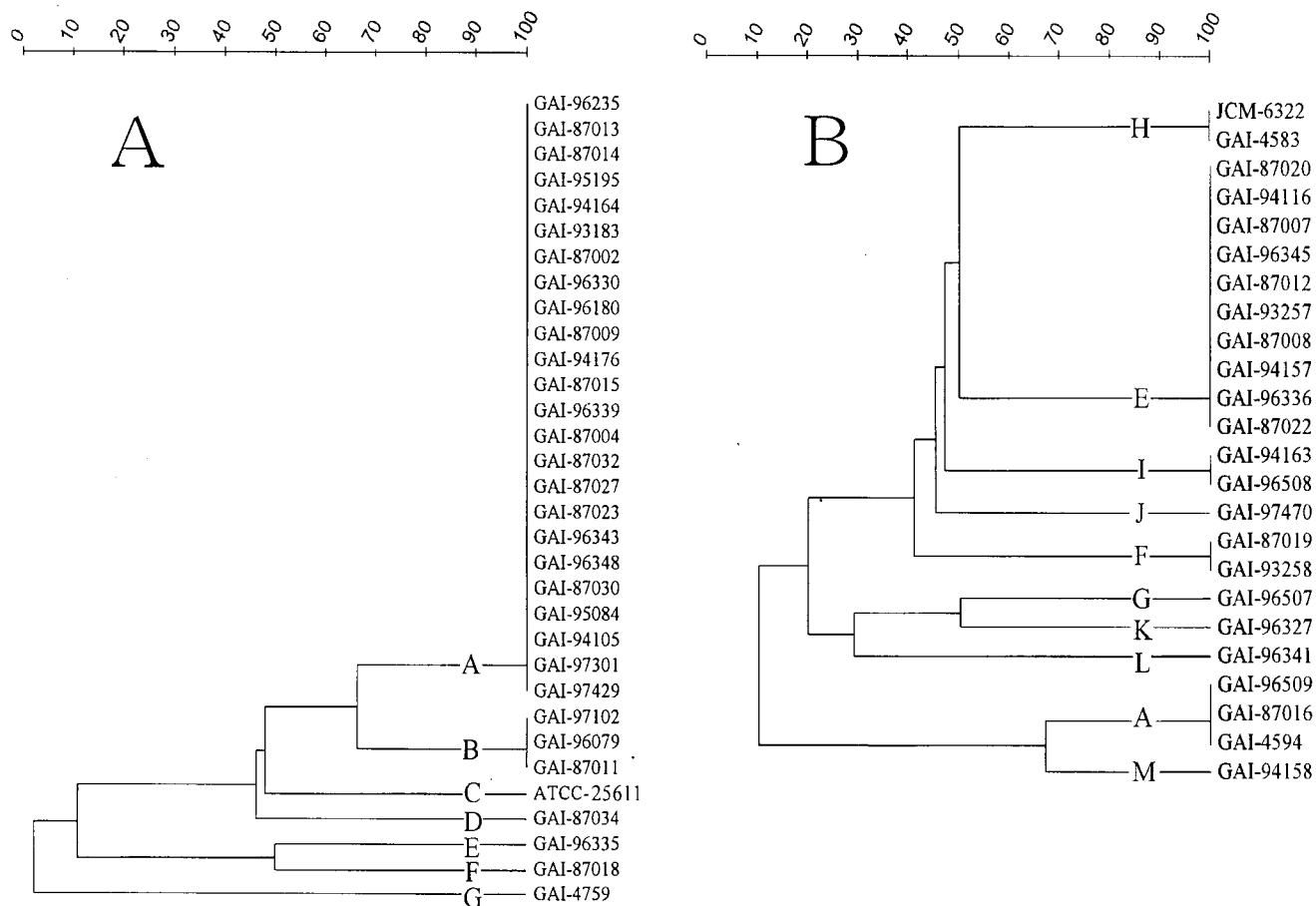


FIG. 2. Dendrograms of PCR ribotyping results for *P. intermedia* ATCC 25611 and 31 clinical strains of *P. intermedia* (A) and *P. nigrescens* JCM 6322 and 23 clinical strains of *P. nigrescens* (B). Each type was designated by a letter from A to M. Types A, E, F, and G were found in two species.

were grouped into 8 subtypes and 3 type A, 2 type F, 2 type H, and 2 type I strains were all discriminated into separate subtypes (data not shown). As a result, the discrimination index of PCR ribotyping followed by AP-PCR was up to 0.99 for both *P. intermedia* and *P. nigrescens*.

Typing and subtyping of *P. intermedia* and *P. nigrescens* strains isolated in family study. Mothers, fathers, and children of 18 families were tested for carriage of *P. intermedia* sensu lato. In 6 of the 18 families tested, all three family members were found to carry *P. intermedia* sensu lato. Thus, the 18

subjects in the six families were analyzed further (Table 1). The ages of the mothers and fathers studied ranged between 36 and 49 years (mean, 42.6 years), while the ages of the children were between 4 and 15 years (mean, 11.0 years).

A total of 293 strains of *P. intermedia* sensu lato were isolated from 18 subjects and were identified by the PCR method; 115 were *P. intermedia* and 178 were *P. nigrescens*. The number of isolates per subject varied between 5 and 20 (mean, 16.3). Of the 18 subjects, 7 (39%) harbored both species.

These isolates were first typed by the PCR ribotyping method (Table 1). Of the 115 *P. intermedia* strains isolated, 93 were type A, 1 was type E, 1 was type L, and 20 were type O, while of the 178 *P. nigrescens* strains isolated, 2 were type A, 161 were type E, 1 was type H, 1 was type L, 3 were type N, 8 were type P, 1 was type Q, and 1 was type R. Of 18 subjects, 12 (67%) carried more than one type of the same species. *P. intermedia* type A was recovered from 10 subjects (56%) and types E, L, and O were recovered from only 1 subject each, while *P. nigrescens* type E was found in 13 subjects (72%) and types A, H, L, N, P, Q, and R were recovered from 1 subject each.

To clarify family-to-family variations in *P. intermedia* and *P. nigrescens* strains, AP-PCR fingerprinting was carried out to subtype the strains (Table 1); type A, the most predominant type of *P. intermedia*, was subtyped into 7 subtypes (subtypes A1 to A7), and type E, the most predominant type of *P.*

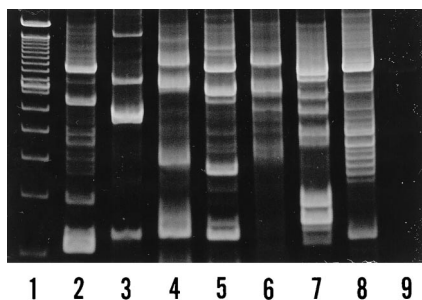


FIG. 3. AP-PCR patterns of *P. intermedia* type A strains. Lane 1, 100-bp DNA ladder; lanes 2 to 8, subtypes A1 to A7, respectively; lane 9, negative control without DNA.

TABLE 1. Occurrence of *P. intermedia* and *P. nigrescens* in family members with adult periodontitis and simple childhood gingivitis^a

Family no.	Member	Age (yr)	Gender	Periodontal status	Sampled tooth	Type and subtype (no. of strains) ^b		Total no. of strains tested
						<i>P. intermedia</i>	<i>P. nigrescens</i>	
1	Mother	47	F	AP	U-F	A1 (16), A2 (2), E1 (1)		19
	Father	49	M	AP	U-F	A1 (17)	E1 (2)	19
	Child	4	M	G	U-E	A1 (2)	N1 (3)	5
2	Mother	42	F	AP	L-FP	A3 (17), A4(1)	E2 (1), H1 (1)	20
	Father	45	M	AP	U-C	A4 (1)	E3 (7), E4 (6)	14
	Child	12	F	G	L-FP	A4 (17)		17
3	Mother	42	F	AP	U-F	A5 (10)	E5 (1)	11
	Father	43	M	AP	U-F	O1 (20)		20
	Child	13	M	G	U-C	A5 (1)	E6 (19)	20
4	Mother	36	F	AP	U-F		E7 (19)	19
	Father	41	M	AP	U-FP	A6 (1), L1 (1)	A8 (2), E7 (15)	19
	Child	12	M	G	U-I		E7 (19), L1 (1)	20
5	Mother	43	F	AP	U-FP		E8 (16), E9 (1)	17
	Father	46	M	AP	U-F		E8 (18), E9 (2)	20
	Child	15	F	G	U-F		E8 (5), E9 (1)	6
6	Mother	37	F	AP	L-S	A7 (8)		8
	Father	40	M	AP	U-FP		E10 (10), P1 (8), Q1 (1), R1 (1)	20
	Child	10	F	G	U-S		E10 (19)	19
Total no. of strains tested						115	178	293

^a Abbreviations: M, male; F, female; AP, adult periodontitis; G, simple gingivitis; U, maxilla; L, mandibular; F, first permanent molar; E, second deciduous molar; FP, first premolar; C, central incisor; C, cuspidate tooth; I, lateral incisor; S, second premolar.

^b Types designated A to R were determined by PCR ribotyping. Subtypes determined by AP-PCR are designated with numeral following the type denoted with a letter.

nigrescens, was subtyped into 10 subtypes (subtypes E1 to E10). As representative results, the results of PCR ribotyping and AP-PCR for family 2 (Table 1) are shown in Fig. 4. Eventually, *P. intermedia* was isolated from five families and 11 subjects and *P. nigrescens* was recovered from six families and 15 subjects, but the same subtypes were never shared by different families (Table 1). The same subtypes were shared by two pairs of spouses for *P. intermedia* (Table 1, families 1 and 2) and two

pairs of spouses for *P. nigrescens* (Table 1, families 4 and 5). The same subtypes were shared by a parent and the child of three families for *P. intermedia* (Table 1, families 1, 2, and 3) and three families for *P. nigrescens* (Table 1, families 4, 5, and 6). Multiple subtypes were found in 8 (57%) of the 14 *P. nigrescens*-positive subjects and 3 (27%) of the 11 *P. intermedia*-positive subjects, but the difference was not statistically significant ($P = 0.14$).

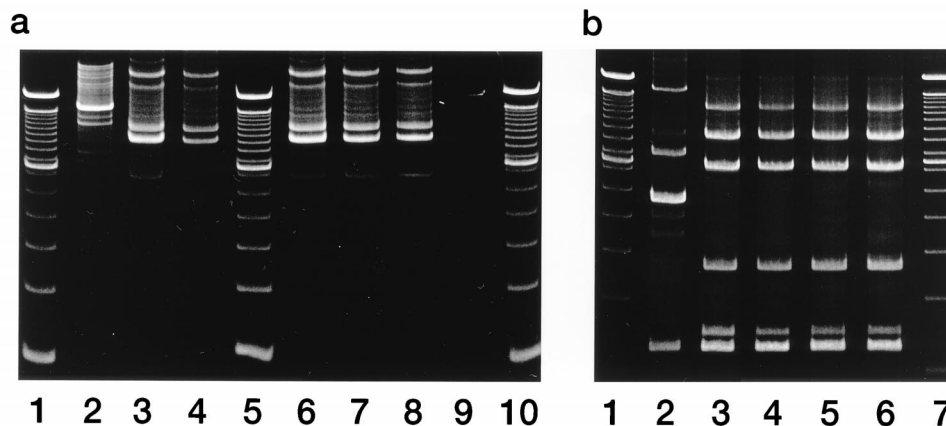


FIG. 4. Results of PCR ribotyping (a) and AP-PCR (b) for *P. intermedia* strains isolated from family 2. (a) Lanes 1, 5, and 10, 100-bp DNA ladder; lanes 2 and 3, isolates from the mother; lane 4, an isolate from the father; lanes 6 to 8, isolates from the child; lane 9, negative control without DNA. (b) Lanes 1 and 7, 100-bp DNA ladder; lanes 2 and 3, isolates from the mother; lane 4, an isolate from the father; lanes 5 and 6, isolates from the child.

DISCUSSION

PCR ribotyping, which demonstrates variations in the length and the number of the 16S-23S rRNA gene spacer regions of microorganisms, has been used to type several bacterial species (1, 8, 13) since this technique gives rather stable typing results on repeat testing and simple banding patterns and thus allows the comparison of the results obtained by separate runs. However, in this study PCR ribotyping proved not to be a highly discriminatory method for strains of *P. intermedia* and *P. nigrescens*. Meanwhile, AP-PCR for these organisms gave reliable typing results with significant discriminatory power in a run but had run-to-run variations (data not shown). Thus, in this study PCR ribotyping was applied to group strains into types, and then strains were subtyped by a separate AP-PCR run for each type. As a consequence, PCR ribotyping followed by subtyping by AP-PCR fingerprinting for isolates from patients with extraoral infections had an excellent discrimination power of 0.99 for both *P. intermedia* and *P. nigrescens* species. This system seems to provide valuable information on the predominant PCR ribotypes of *P. intermedia* and *P. nigrescens* in a population and to be highly discriminating for strains of these two species.

On the basis of these results, the combination of PCR ribotyping and AP-PCR was applied to the study of intrafamilial carriage of *P. intermedia* and *P. nigrescens*. Our data indicate that a *P. intermedia* or *P. nigrescens* strain(s) of the same subtype can colonize spouses or parents and children. The results are compatible with those of previous studies (11, 20).

Recently, studies have reported that *P. intermedia* seems to be associated with advanced periodontitis and that *P. nigrescens* is likely to be predominant at healthy gingival sites of children (11, 20). This study demonstrated that both *P. intermedia* and *P. nigrescens* were predominant in adults and children who had subclinical early-stage to moderate periodontal disease for which no treatment but tooth brushing was required. To draw conclusions about the pathogenicities of these species, a comparative evaluation of the incidence of *P. intermedia*, *P. nigrescens*, and another established periodontal pathogen such as *Porphyromonas gingivalis* should be performed on the basis of the stage of periodontal disease.

In conclusion, the combination of PCR ribotyping and AP-PCR fingerprinting proved to be well suited for the epidemiological study of *P. intermedia* and *P. nigrescens*. The same genotype(s) of both species can colonize spouses or parents and children.

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